Characterization of antibacterial drugs and drug combinations targeting oxidative phosphorylation in Mycobacteria

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SUMMARY AND GENERAL DISCUSSION

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Tuberculosis (TB) remains a major global health problem, despite so much effort has been put into combating this disease. In particular, the emergency of multidrug-resistant TB (MDR-TB) and extensively drug-resistant TB (XDR-TB) as well as co-infection with HIV poses a serious challenge. Moreover, the flexibility of *M. tuberculosis* metabolism and heterogeneity of bacterial populations makes the treatment of TB more complicated. Therefore, new drugs with novel working mechanism are desperately needed to adequately kill the heterogeneous population of bacteria and to counter MDR and XDR tuberculosis. In recent years, energy metabolism has emerged as a new target for development of antimycobacterial drugs[1] and the identification of new candidate drugs targeting energy metabolism illustrates the therapeutic potential of blocking mycobacterial energy conversion[2, 3].In this thesis, we expanded the knowledge of energy metabolism in mycobacteria as drug target. We investigated the mechanism of a presently used front-line drug, tested new drug combinations, described a recently found target and explored a new potential target. The knowledge acquired in this thesis may contribute to the identification of putative important factors in mycobacterial energy metabolism, which might lead to the discovery of new antimycobacterial drug targets.

The special features and adaptions of mycobacterial energy metabolism

As mycobacteria encounter a challenging environment in the human host, these bacteria may carry special features in energy metabolic pathways. This may include both usage of bacteria-specific components of oxidative phosphorylation as well as adaptations in complexes that do have homologues in human metabolism. As an example for the first case, mycobacteria, unlike most eukaryotes cells, employ two types

of NADH dehydrogenases as electron donors for oxidative phosphorylation. Type I NADH dehydrogenase (NDH-1) is preferably used under aerobic conditions[4],while NDH-2 is favored under anaerobic or non-replicatingconditions[5]. Mutagenesis studies have shown thatNDH-1 is dispensable for *M. tuberculosis* growth *in vitro* and mutations in NDH-2 led to a lethal phenotype[6, 7]. All of these evidences imply that NDH-2 is the dominant NADH dehydrogenase involved in mycobacterial energy metabolism. Inhibitors that specifically target NDH-2, such as the phenothiazine class of drugs, show bactericidal activity and may have good drug potential for drug synergy, suggesting that this key enzyme presents a potential new drug target.

Mycobacteria also display a branched respiratory chain with two terminal oxidases, the aa_3-type cytochrome c oxidase (connected to the cytochrome bc_1reductase complex) and the cytochrome bd oxidase. The aa_3-type cytochrome c oxidase, which apparently forms supercomplex with the cytochrome bc_1 reductase, is more energy efficient than the cytochrome bd route and it is the major respiratory route under standard, aerobic culturing conditions[8]. There is little known about potential special features in the cytochrome bc_1 complex or in the aa_3-type cytochrome c oxidase. However, gene sequence analysis indicate that mycobacteria, like *C. glutamicum* or *R. rhodochrous*[9],have a distinct, diheme c-type cytochrome c_1(QcrC)[10]. It would be interesting to investigate the biochemical properties of this fused protein, most probably functioning as a connection between the cytochrome bc_1 complex and the cytochrome c oxidase. Moreover, comparing the capability of generating proton motive force between the mycobacterial bc_1-aa_3supercomplex and homologous complexes from other bacteria could be another important step. The cytochrome bc_1complex in *M. tuberculosis*has been validated as target of the imidazo[1,2-a]pyridine amide (IPA)

drug class, of which Q203 is a promising candidates to treat tuberculosis[11, 12].

The cytochrome bd oxidase on the other hand is less well investigated and its function may be complex. The cytochrome bd branch is less energy efficient and it seems to play an important role under reduced oxygen conditions[13, 14]. Our results (Chapter 6) demonstrated that cytochrome bd, although not required for aerobic growth, plays an important role in response to antibacterial stress. Targeting cytochrome bd therefore appears highly promising in order to weaken bacterial metabolism and for efficient drug combination regimen. Structural and functional analysis of cytochrome bd is strongly needed to exploit this enzyme as a drug target.

*M. tuberculosis*is an obligate aerobic bacterium, as such, it would be dependent on oxidative phosphorylation to produce ATP for growth and survival. ATP synthase is evolutionarily strongly conserved among prokaryotes and eukaryotes cells. Since ATP synthase is essential not only in replicating mycobacteria, but also in the dormant state[5, 15], as such ATP synthase in these mycobacteria may carry special features that facilitating survival under these dormant conditions, including low oxygen tensions, nutrient limitation or residinginside human macrophages.

The mycobacterial ATP synthase lacks the ATP hydrolysis function, can synthesize ATP under low proton motive force (~ -100 mV), and displays an unusual stator stalk. Thesespecial features may help mycobacteria adapting to theexceptional challenges of theirenvironment. However, special features present in the amino acid sequence of mycobacterial ATP synthase need to be linked to the function of this enzyme. Mutagenesis or other experimental studies need to be carried out to determine the phenotype associated with unique features in mycobacterial ATP synthase.

Although ATP synthase appears to be present in all bacteria, the essentiality of the enzyme varies across species. For mycobacteria, the deletion mutants in ATP synthase genes result in loss of optimal growth both in *M. tuberculosis* and in *M. smegmatis*[6, 16]. Moreover, small molecule inhibitors, which targeting the ATP synthase or interfering with ATP synthesis have bactericidal effects against mycobacteria[17]. For *E. coli* and *B. subtilis*, mutations in ATP synthase only result in a slowing growth rate comparing with the wild type[18, 19]. Apparently, the increased glycolytic metabolism may in part compensate for the deficiencies in respiratory ATP synthesis. For other pathogenic bacteria, such as *Staphylococcus aureus*, deletion mutants in atpD, atpG, atp A and atpE genes lost strong but not completely bacterial viability[20, 21].In addition, the diarylquinoline class of inhibitors, which targets the ATP synthase enzyme, inhibits the growth of *S. aureus* in planktonic state as well as in biofilm model[21]. Small molecule inhibitors targeting ATP synthase may thus be not onlyrestrictedto mycobacteria, but may also be applicable to a broader spectrumof pathogenic bacteria. For this reason it would be very interesting to further study the working of the respiratory chain and of ATP synthase in biofilm settings. Moreover, sequence analyses and functional studies of ATP synthase form *S. aureus* or other Gram-positive strains may reveal if these pathogens, like mycobacteria, carry special features in this central enzyme.

The strategy of targeting components of oxidative phosphorylation, in spite of its homologue within eukaryotic cells, may present a new way for the discovery of novel drug targets.

Synergy between bedaquiline and pyrazinamide, a possible backbone for future anti-TB regimen.

In this thesis, we found synergy between bedaquiline(BDQ) and pyrazinoic acid against *M. bovis* BCG. Previously, Ibrahim *et al* had shown that the combination of BDQ and pyrazinamide (PZA)has exceptional bactericidal activity in a murine TB model[22]. Later, the same group demonstrated that regimen containing BDQ+PZA allow for reduction of TB treatment duration from 6 months to 4 months in the murine model[23]. In the meantime, Tasneen *et al* showed that the 2-drug combination BDQ+PZA given for 3 months was superior to 4 months (and equivalent to 5 or 6 months) of the standard regimen concerning prevention of TB relapse in mice[24]. For drug resistant tuberculosis (DR-TB) treatment, the BDQ plus PZA regimen also showed promising results. Veziris *et al* reported that the combination BDQ+PZA+moxifloxacin can reduce the duration of MDR-TB treatment to 6 months as compared with 12 months for the standard regimen[25]. Why does this drug combination displaysuch a strong synergetic effect? The synergy might due to several reasons. First, factors associated with the mammalian host may play a role in the mutual interaction of the two drugs. However, we found synergy in an *in vitro* system, clearly revealing that the synergy at least in part due to mycobacterial factors. Second, PZA is also reported to inhibit ribosomal trans-translation under stress conditions[26]. The importance of trans/translation may be enhanced under BDQ-induced stress condition, due to reduced cellular ATP levels. This mechanism may further contribute to the synergy between two drugs. Third, the enhanced kill by BDQ+PZA correlated with an increased drop of cellular ATP (chapter 4).This drop may inactivate ATP-driven multidrug efflux pumps, thereby indirectly accumulating more PZA in the cell. Synergy with PZA has also been reported for a second ATP synthase inhibitor,

dicyclohexyldiimide (DCCD), supporting this hypothesis[27]. Moreover, in line with this hypothesis we found in chapter 5 that BDQ inactivates efflux of a small-molecule, ethidium bromide, in a mycobacterium. These experiments may be expanded to determine if inhibitors of energy metabolism in drug combinations enhance each other's potency by inactivating the efflux of the respective companion drug. This can be further investigated for the drug combination of BDQ+PZA with clofazimine (CFZ), which interferes with NDH-2[28]. The regimen BDQ+PZA+CFZ showed 7% relapse rate after 6 weeks treatment in a murine TB model, clearly lower than BDQ+PZA with rifapentine (RPT), a DNA-dependent RNA-polymerase inhibitor (33%)[29].

In summary, energy metabolism inhibitors show strong synergy in drug combinations, likely at least in part due to inactivation of efflux pumps. This synergy may contribute to current efforts on shortening TB chemotherapy. A regimen based on BDQ+PZA+one of the second/third line anti-TB drug can have a strong sterilizing activity after 3-month treatment[24].Moreover, BDQ+PZA+RPT and BDQ+PZA+CFZ can even reduce this period to 2 months[24, 30].

RIF and RPT may significantly decrease other drugs' therapeutic concentrations in human through cytochrome P450 enzyme induction, for instance, RIF reduces approximately 50% of BDQ levels in plasma[31]. Therefore, in the future we may replace RIF with a second line anti-TB drug. The new regimen selection may have to compromise with other pipeline drug candidates, but the backbone of the new regimen should be BDQ+PZA, because of its superior bactericidal and sterilizing activity. A regimen based on this backbone combination plus one of the pipeline drug candidates can significantly reduce the treatment time to 2 or 3 months. The exact treatment duration estimates need to be confirmed in clinical trials,

results from a 14-day clinical trial with the combination BDQ+PZA were strongly encouraging[32].

Targeting energy metabolism for treating persistent infection

Persistent infections comprise replicating or non-replicating bacteria, which are difficult to treat with conventional antimicrobials. As a result, there is an urgent need for antimicrobials that can treat infections containing persistent dormant bacteria. However, most of the currently used antibiotics were developed by screening bacteria under favorable replicating conditions, and most target five biosynthetic processes such as the biosynthesis of proteins, RNA, DNA, peptidogly can and folic acid[33]. Therefore, most of these classical antimicrobial strategies are not effective for eradicating persistent infections in which bacteria are quiescent (hypoxia, non-replicating or dormant). *M. tuberculosis* residing in the patients' lung forming a latent tuberculosis infection model is one of the clinical examples. In general, these cases require prolonged treatment periods to cure or alleviate the burden of disease. Consequently, new antimicrobial strategies, which could shorten the treatment period, reduce disease relapse will be needed to cure infections that contain quiescent bacteria.

In our opinion, antimicrobials that target energy metabolism are promising therapeutic approaches for treating non-replicating or dormant bacterial infections. Persistent bacterial subpopulations need to adapt to various environments in the host during this kind of infection. During this adaptation, bacteria have to face unfavorable conditions such as host responses, low pH, nutrient and oxygen deprivation. Although bacteria have remodeled key metabolic

pathways, in particular biosynthesis pathways[34, 35], they still require ATP and redox balancing to survive. Inhibitors of energy metabolism that bind directly to target enzymes in the membrane that are involved in energy conversion and in the NAD^+/NADH redox balance seem to have strong bactericidal activity against persisting bacteria[3, 5, 15]. Even though this strategy is a new direction for antibiotic discovery, there are several challenges. First, bacteria may have more than one way to compensate their energy metabolism loss when they encounter unfavorable conditions. For example, ATP synthase is unessential for staphylococci; they can survive solely by increased activity in fermentation using the glycolytic pathway and they can persist in the host for prolonged periods[36]. Second, persistent infections can involve heterogeneity of bacterial subpopulations; a failure to kill all subpopulations will demand an additional drug to sterilize the infection.

At present, the spectrum of antibacterials blocking energy metabolism to treat persistent infection is not only restricted to mycobacteria, but also expanded in other bacterial strains. Inhibition of cytochrome oxidase in *P. aeruginosa* results in reduced growth rate and poor biofilm formation under aerobic conditions[37]. The diarylquinoline class of small molecules interferes with cellular ATP homeostasis in *S. aureus* or *S. pneumonia*, therefore inhibitingthe bacteria growth in planktonic state as well as in metabolically resting state in a biofilm culture[20].

In summary, we anticipate that as this exciting field progresses, more and more novel agents will be discovered that could shorten treatment periods and improve clinical outcomes for persistent infections.

How to search for new chemical scaffolds for combating tuberculosis?

The gap between the global tuberculosis burden and the discovery of new chemical scaffolds is still very large. The approval of bedaquiline for treating multidrug resistant tuberculosis by the U.S FDA in 2012 is one of the few success stories[38]. The poor efficiency of identifying new TB drugs might due to several reasons. First, the traditional pharmaceutical library collections for high-throughput screening are too limited. Therefore, we cannot find enough new chemical compounds that have bactericidal activity against *M. tuberculosis*. Second, some compounds can inhibit an essential target, but do not have enough permeability to penetrate into the highly impermeable mycobacterium cell or may have unfavorable pharmacokinetics. In spite of these challenges, the current TB drug development pipeline is slowly expanding. Maybe we can get some useful indication from current drug scaffolds, which may led us to find new chemicals for combating tuberculosis.

In particular, the thick mycobacterial cell envelope can form an important obstacle for drug influx. For example, in this thesis we found that the small molecule aurachin D could inhibit cytochrome bd in mycobacterial membrane vesicles at nanomolar concentrations (chapter 6). However, this molecule cannot inhibit the mycobacterial growth. To address this problem we have to change our screening strategy from essential-enzyme targets to a whole bacterial cell level. This strategy recognizes all interactions of a drug, which may interfere with one or more components of a bacterial cell. One of the drawbacks of the whole cell screening is that the knowledge regarding the mechanism of action is largely unknown. Secondly, the metabolic heterogeneity of bacteria in *vivo* may reflect different drug susceptibility, which requires

identification of the right *in vitro* growth conditions for the whole cell screening. Moreover, some hits from whole cell screening need to be excluded as artifacts, such as detergent effects and cytotoxic effects via non-specific mechanism. The recent success with whole cell screening is exemplified by the identification of bedaquiline and the benzothiazines(BTZ043). Bedaquiline is now a new proved MDR-TB drug; BTZ043 is still in the early drug discovery phase.

Another new anti-TB drug search approach is reengineering of old drug classes that were discovered decades ago[39]. During reengineering of known scaffolds, chemical modifications are introduced into the core structure, which may lead to improved bactericidal activities, better resistance profiles, and superior pharmacokinetic properties. This approach has either identified new TB drugs from redesigned old chemicals to improve their antimycobacterial activity or relocating of known antibacterials drugs for testing in TB clinical trials. For instance, the oxazolidinones chemical class was used to treat Gram-positive infections, but the modified products such as PNU-100480 and AZD-5847 have significant activity against *M. tuberculosis*[40]. These oxazolidinone TB drug candidates, currently in phase I studies, still need to meet many clinical development requirements before they can be used for TB treatment. Another example is the nitroimidazoles, traditionally used to treat anaerobic bacteria and parasitic infections. PA-824 and OPC-67683, two candidates from this class with modified structure, have great potential to shorten TB treatment duration as they are active against dormant mycobacteria[41]. Although gradual improvements of existing scaffolds is a good strategy to fill the gap between tuberculosis burden and drug shortage, the increasing resistance to some of these existing drug classes indicates that discovery of new chemical scaffolds is a more attractive approach. New

chemical scaffolds with novel working mechanism definitely need to investigate in the future.

Concluding remarks

The work presented in this thesis has provided insight in exploiting the new concept of energy metabolism as drug target in anti-TB drug development. We started out with characterizing the mechanism of a long-known front line anti-TB drug. Subsequently, we investigated new drug combinations acting on energy metabolism, analyzed the features of ATP synthase as a recently found target and explored cytochrome bd as a new potential target. Results obtained from these experiments might help us pinpoint components of energy metabolism as Achilles' heel of mycobacterial metabolism. Altogether, these studies provide more information on using energy metabolism as drug target, which may aid in an effective drug discovery and to a better TB treatment.

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